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Genetic engineering of *Penicillium chrysogenum* for the reactivation of biosynthetic pathways with potential pharmaceutical value

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CHAPTER 1

INTRODUCTION

FUNCTIONAL ANALYSIS OF POLYKETIDE GENE CLUSTERS IN *PENICILLIUM CHRYSOGENUM*

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In Preparation

I. *PENICILLIUM CHRYSOGENUM* AND SECONDARY METABOLISM

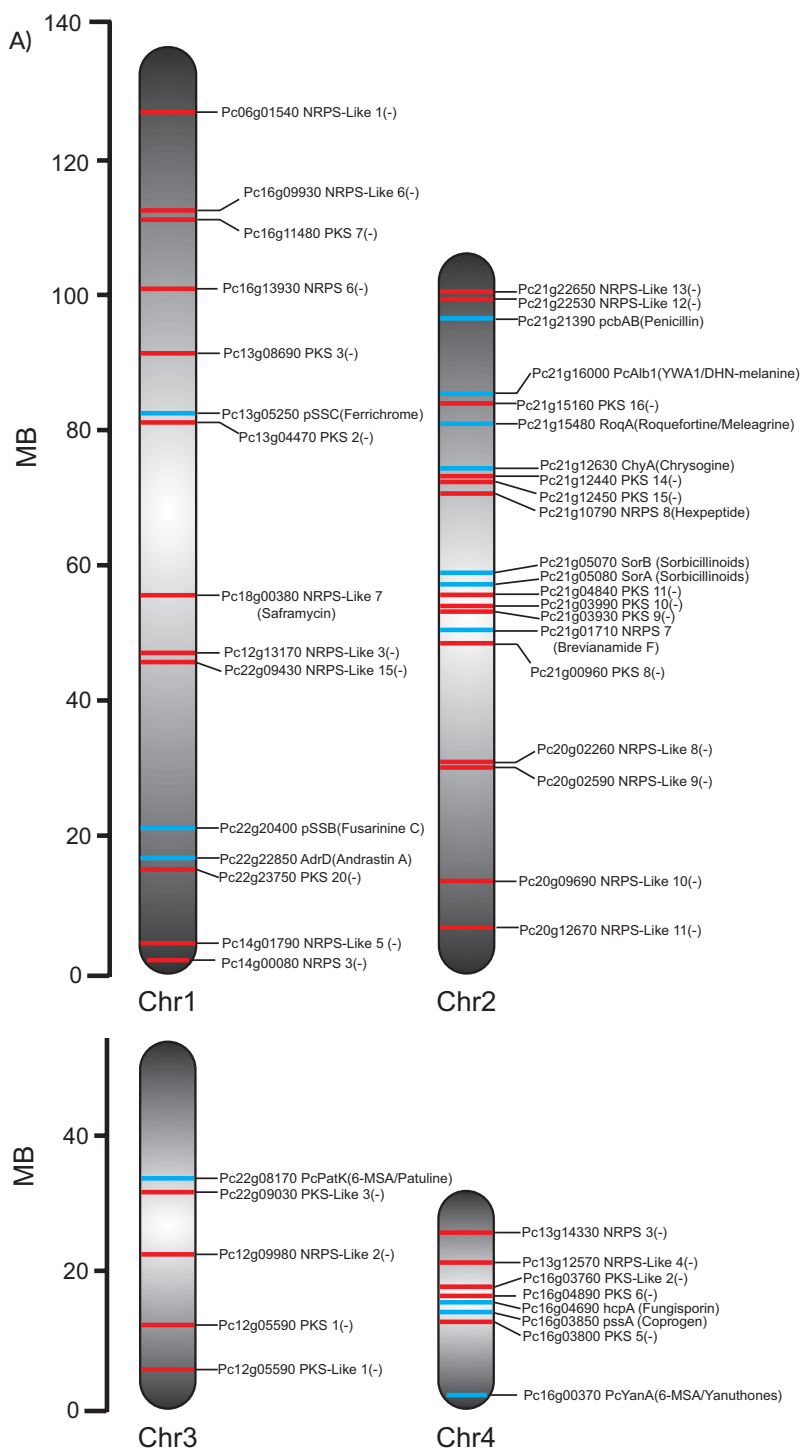
Since the discovery of penicillin by Alexander Fleming in the filamentous fungus *Penicillium notatum*, the genus *Penicillium* has been deeply studied due to its capacity to produce a wide range of secondary metabolites, many of them with biotechnological and pharmaceutical applications. *P. chrysogenum* (also identified as *P. rubens*) is the most relevant member of more than 354 species that integrate the genus (Nielsen *et al.*, 2017). This species is usually found in indoor environments and associated with food spoilage. It is known as an industrial producer of β -lactams in particularly penicillin, and current production strains result from several decades of classical strain improvement (CSI) (Gombert *et al.*, 2011; Houbraken *et al.*, 2011). The CSI program began in 1943 with the isolation of *P. chrysogenum* NRRL 1951 capable of growing in submersed cultures. This strain was subjected to a long serial process of single spore selection, mutations induced by 275 nm ultraviolet and X-ray irradiation, nitrogen mustard gas and nitroso-methyl guanidine exposure and selection for loss of pigments and improved growth in large scale industrial fermenters. CSI programmes were developed in several companies (Barreiro *et al.*, 2012), and this has resulted in an increase of penicillin titers by at least three orders of magnitude (van den Berg, 2010). As consequence, numerous genetic modifications were introduced in *P. chrysogenum*. Some have been studied in detail, most notably the amplification of the penicillin biosynthetic clusters and DNA inversions in this region (Barreiro *et al.*, 2012). Although the CSI had a major impact on the production of β -lactams by *P. chrysogenum*, it also affected other secondary metabolism. Genome sequencing of *P. chrysogenum* Wisconsin 54-1255 revealed the presence of several further secondary metabolite gene clusters in addition to the penicillin cluster, most of which have poorly been studied and remain to be characterized (Figure 1). The products of the gene clusters are either nonribosomal peptides, polyketides or hybrid molecules.

P. chrysogenum produces a broad range of secondary metabolites such as roquefortines, fungisporin (a cyclic hydrophobic tetrapeptide), siderophores, penitric acid, ω -hydroxyemodin, chrysogenin, chrysogine, sesquiterpene PR-toxin and sorbicillinoids, but likely also possesses the

ability to produce compounds not detected before. However, for most of these compounds, the responsible genes are unknown. The development of new bioinformatics tools (SMURF, AntiSMASH) and the increase in the number of fungal genomes sequenced has opened the possibility to discover new natural products with novel properties (genome mining). The genes involved in the biosynthesis, regulation and transport of secondary metabolites tend to be arranged in the genome in Biosynthetic Gene Clusters (BGCs). Importantly, these gene clusters include the core biosynthetic genes which either encode polyketide synthases (PKSs), non-ribosomal peptide synthetases (NRPSs) or terpene synthases genes (Smanski *et al.*, 2016). Recently, a global analysis was performed on 24 genomes of *Penicillium* species and this identified 1317 putative BGCs predominating subdivided in two classes based on PKS (467) and NRPS (260) (Nielsen *et al.*, 2017). In *P. chrysogenum* there are 33 core genes that encode 10 NRPS, 20 PKS, 2 hybrid NRPS-PKS and 1 dimethyl-allyltryptophan synthase (van den Berg *et al.*, 2008; Khaldi *et al.*, 2010; Medema *et al.*, 2011; Samol *et al.*, 2016) (Figure 1). Large number of PKS and NRPS enzymes are found also in other *Penicillium* species but only part of these gene clusters overlap, which suggests an unexplored potential of the secondary metabolome even in a single genus. Here, we discuss the individual BGCs that have been identified and characterized in *P. chrysogenum* with a focus on those encoding polyketides.

II. POLYKETIDES AND POLYKETIDE SYNTHASES

Polyketides were discovered in 1883 by James Collie, although the interest in these compounds (enzymes) was revived only as late as the 1950s by the work of Arthur Birch on the aromatic polyketide-6-methyl salicylic acid from *P. patulum*. These molecules are a class of natural products, that may display different types of biological activities such as antibiotic (erythromycin A), antifungal (amphotericin B), immunosuppressant (rapamycin), antitumor (geldanamycin) and hypolipidemic agents (Lovastatin) (Nair *et al.*, 2012; Jenner, 2016; Weissman, 2016). Their assembly process is similar to that in fatty acid biosynthesis, albeit the main difference is the optional full reduction of the β -carbon in the polyketide biosynthesis. The group of enzymes that catalyzes



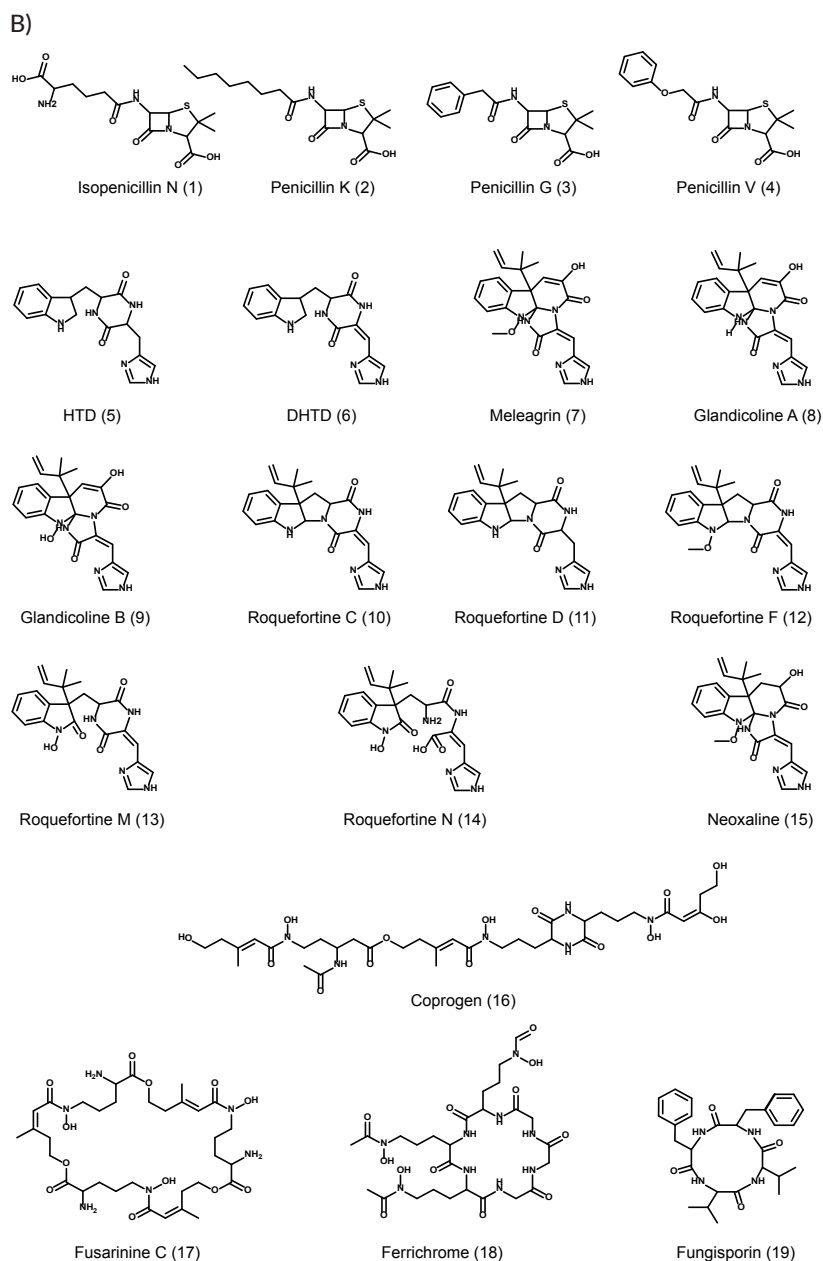
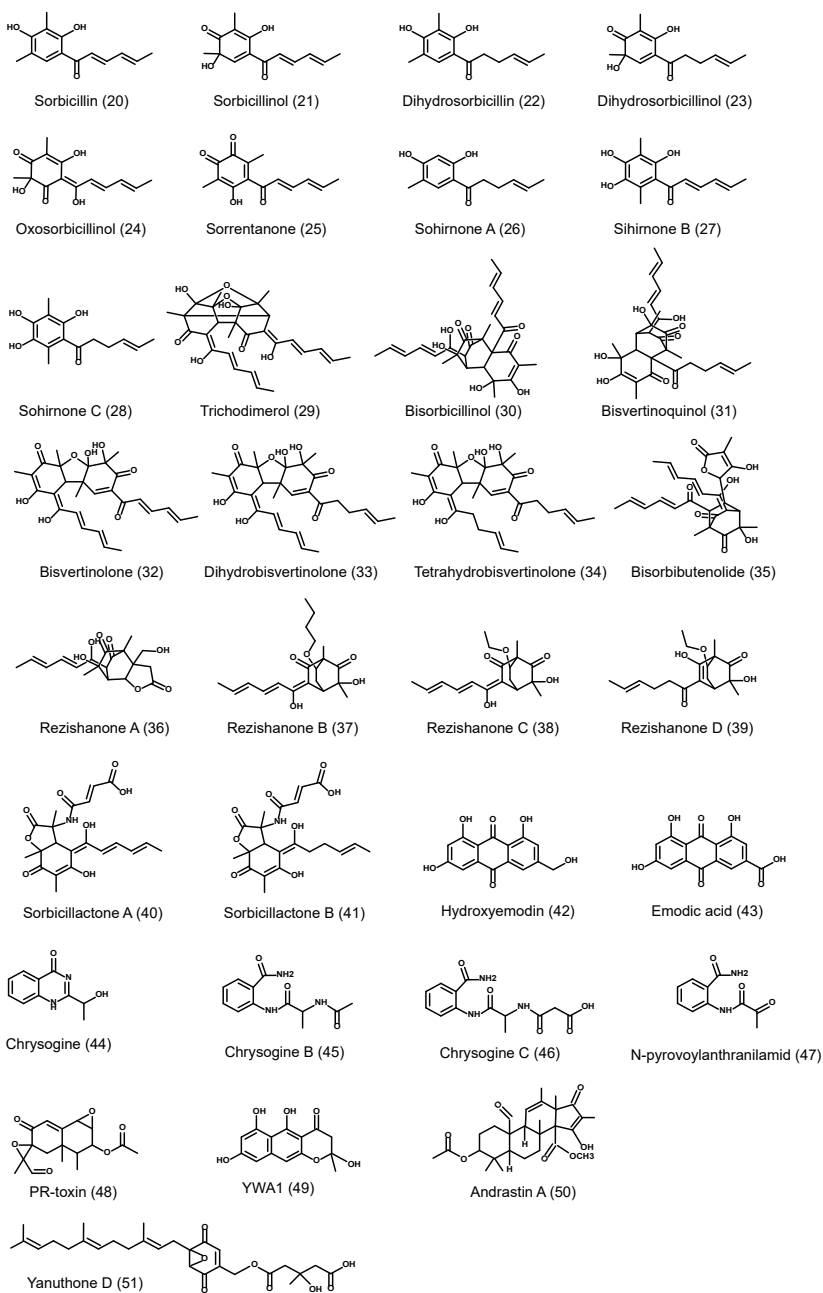


Figure 1. Chromosomal localization of known and predicted PKS and NRPS genes and secondary metabolites identified in *Penicillium chrysogenum*. A) Chromosomal localization of PKS and NRPS genes. Blue and red lines indicate known and unknown associated products so far, respectively. B) Struc-



tures of secondary metabolites produced by *P. chrysogenum*. Adapted from (Ali *et al.*, 2013; Ries *et al.*, 2013; Salo *et al.*, 2015; Salo, 2016; Samol *et al.*, 2016; Guzmán-Chávez *et al.*, 2017).

the biosynthesis of polyketides are referred to as *polyketide synthases* (PKSs) (Keller *et al.*, 2005; Caffrey, 2012).

In addition to the non-ribosomal peptide synthetases (NRPS), PKSs are the main enzymes that build the structural scaffold of a wide range of secondary metabolites and natural products in plants, bacteria, insects and fungi (Brakhage, 2012; Nair *et al.*, 2012). Usually, these enzymes are encoded by genes that are grouped into clusters, that also specify genes encoding tailoring enzymes (oxygenases, oxidoreductases, reductases, dehydrogenases and transferases), that further modify the scaffold produced by the PKS into a final product (Brakhage, 2012; Lim *et al.*, 2012). PKSs are multimodular and multi-domain enzymes that use a specific acyl-coenzyme A (acyl-CoA; usually malonyl-CoA or methylmalonyl-CoA) as building block, and subsequently catalyze a decarboxylative Claisen-type condensation of ketide units (Figure 2). The basic structural architecture consists of an acyl carrier protein (ACP), a ketosynthase (KS) and an acyltransferase (AT) domain. These combined domains extend a linear intermediate by two carbon atoms. An optional set of domains (dehydratase (DH), ketoreductase (KR), enoyl reductase (ER) and thioesterase (TE) may provide further modifications of the linear intermediate (Staunton and Weissman, 2001; Brakhage, 2012; Nair *et al.*, 2012; Dutta *et al.*, 2014).

II.1 ACYLTRANSFERASE DOMAIN (AT)

A main unit during polyketide biosynthesis is the *acyltransferase domain* that selects the start unit (malonyl-CoA or methylmalonyl-CoA) before it is transferred to the ACP domain for the chain elongation cycle (Dunn *et al.*, 2013). This process involves two steps, i.e., the *acylation* and the *transfer to the ACP*. The first step proceeds via nucleophilic attack by a catalytic serine, which is present in the GHSXHG-motif at the thioester carbonyl of the Acyl-CoA. The reaction produces an intermediate and the CoA moiety is released from the active site (Jenner, 2016). During the second step, through a *ping-pong bi-bi mechanism*, the acyl-enzyme (acyl-loaded AT) intermediate is formed, whose ester carbonyl is nucleophilically attacked by the thiol of the phosphopantetheine chain present in the ACP domain (Dunn *et al.*, 2013; Park *et al.*, 2014; Jenner, 2016).

II.2 ACYL CARRIER PROTEIN (ACP)

The ACP is an essential cofactor that participates in polyketide biosynthesis. This protein belongs to a highly conserved carrier family, and consists of 70-100 amino acid residues (Byers and Gong, 2007). Structurally, ACP consists of four α -helices stabilized by a hydrophobic core. A conserved serine residue in the (D/E)xGxDSL motif, that is localized to the N-terminus of helix II, plays an important role in the transition of the ACP from the inactive (apo) to active (holo) form. The *holo*-ACP form is generated by the phosphopantetheinyl transferase enzyme (PPTase) through a post-translational modification of ACP whereby a 4'-phosphopantetheine (4'-PP) moiety from coenzyme A (CoA) is transferred to the conserved serine (Evans *et al.*, 2008; Jenner, 2016) resulting in the formation of the P-pant arm. Due to its negative charge, helix II acts as "recognition helix" over the positive regions on AT and KS domains. Thus, the ACP modulates three important events during polyketide biosynthesis. First, it allows the condensation during chain elongations since it transfers the started unit from the AT domain to the KS domain. Second, it shuttles the growing chain between the up and downstream domains, as well to optional PKS domains, probably involving protein-protein recognition between domains. Third, it prevents premature cyclization and enolization of the polyketide chain (Kapur *et al.*, 2010; Yadav *et al.*, 2013).

II.3 KETOSYNTHASE DOMAIN (KS)

The KS is a homodimeric condensing domain that catalyses the extension of the β -ketoacyl intermediate by a decarboxylative Claisen condensation. This domain contains two active sites that are accessible to the ACP through its flexible P-pant arm, which receives the β -carboxyacyl-CoA extender unit from the AT. At that stage, a thioester bond is formed between the active-site cysteine thiol of KS and the growing polyketide. Only when both units are covalently attached onto the module, the decarboxylative Claisen condensation occurs, which involves two conserved His residues. Therefore, mechanistically the KS domain acts at three stages: acylation, decarboxylation and condensation (Chen *et al.*, 2006; Caffrey, 2012; Yadav *et al.*, 2013; Jenner, 2016; Robbins *et al.*, 2016).

The KS domain may also transfer intermediates between modules without chain extension. This is the case with some KS variants, named KS⁰, that lack the active histidine involved in the polyketide elongation. Another variant is the KS^Q that has a glutamine residue instead of the active site cysteine. This permits loading modules from cis-AT PKS (Jenner, 2016). Recently, a KS domain has been subjected to extensive protein engineering to identify alterations in and around the active site residues of KS, which boosts substrate promiscuity and activity. For instance, in the KS3 domain in the enzyme 6-deoxyerythronolide B synthase (DEBS), a change of an alanine to tryptophan in the active-site proximal dimerization loop increases the promiscuity of the enzyme (Bayly and Yadav, 2017).

II.4 KETOREDUCTASE DOMAIN (KR)

The KR domain functions as a β -carbon processing unit that belongs to the family of short-chain dehydrogenase/reductases. This domain reduces the β -keto group, that is formed during the condensation process, into a hydroxyl group (a β -hydroxyl intermediate) using NADPH (Keatinge-Clay and Stroud, 2006; Caffrey, 2012). Its catalytic activity involves three domains with conserved residues (tyrosine, lysine and serine) and these domains are divided in three types (A, B and C) according to the stereochemical result of the reduction. The A-type produces the levo (L) configuration of the β -alcohol group, while B-type denotes a dextro (D) configuration. The inactive KRs represent the third category, the C-type (Caffrey, 2012; Jenner, 2016; Bayly and Yadav, 2017). Therefore, the stereo configuration of the α - and β -carbon atoms of ACP substrates is dictated by the KR domain. Additionally, some KR domains are equipped with epimerase activity. The epimerizing module has a more open architecture, enabling the catalytic epimerization of methyl groups in acyl-ACP substrates, a reaction that involves the conserved serine and tyrosine residues that are also employed during ketoreduction (Ostrowski *et al.*, 2016; Bayly and Yadav, 2017). However, it is not obligatory that both activities, ketoreduction and epimerization, are present in the KR domain. Indeed, some KR domains only have epimerase activity (C2), while others only show ketoreductase activity (A1 and B1), both activities (A2 and C2)

or are catalytically inactive (C1) (Annaval *et al.*, 2015). KR domains are promising targets for engineering since their signature motifs can be used to predict the stereochemistry of the KR domains, albeit there are some exceptions. The most successful method for KR engineering is modification of the active site or swapping of the reductive loop (RLS) (Kellenberger *et al.*, 2008).

II.5 DEHYDRATASE DOMAIN (DH)

The DH domain is usually coupled to B-type KR domains (B-type). This domain catalyzes water elimination (via *syn* or *anti*) at the β -hydroxy acyl chain position thereby producing *trans* double bonds (α,β -unsaturated moieties). It has been proposed that an aspartic acid located in the HPALLD motif is the proton donating entity whereupon the catalytic histidine, in the HXXGXXP motif, eliminates an α -proton, resulting in unsaturation. However, when the dehydration occurs on A-type ketoreduction products ((3*S*)-3-hydroxyacyl chain) a *cis* double bond is formed. The diastereospecificity nature of this domain is an important target for engineering. Structurally, the DH domain consists of a central helix and seven β sheets which are arranged in a double hotdog fold, with one active site. This differs from the DH domains of fatty acid synthases (FAS) DHs that may have many active sites per double hotdog fold (Caffrey, 2012; Bruegger *et al.*, 2014; Jenner, 2016; Bayly and Yadav, 2017).

II.6 ENOYLREDUCTASE DOMAIN (ER)

The ER domain is an optional tailoring unit involved in the final oxidation state of the growing polyketide. It reduces α,β -enoyl groups and thereby generates saturated $\alpha-\beta$ bonds. This reaction involves NAD(P)H as hydride donor in Michael addition type of mechanism. In the enoyl reduction, the products formed during this reaction have a specific stereochemistry (3*R*,2*R*) or (3*R*,2*S*) due to the β -carbon attack performed by the pro-4*R* hydride of NADPH, contrasting the KR domain that utilizes the pro-4*S* hydride (Chen *et al.*, 2006; Bruegger *et al.*, 2014). Structurally, the ER domain belongs to the medium-chain dehydrogenase/reductase (MDR) superfamily of enzymes. In contrast to

monomeric state presented in the *trans* ER domains, highly reducing ER domains form dimers.

Additionally, it has been reported that the stereospecificity of the S-configuration depends on the presence of a tyrosine in the ER domain that is used for the protonation of α -carbon yielding (2S)-alkyl-branched products. Indeed, (2R) products are detected when the active tyrosine is absent or replaced by alanine, valine or phenylalanine (Kwan and Leadlay, 2010; Bruegger *et al.*, 2014). In fungi, the wide range of polyketide products mostly emerges from *trans*-acting ER domain. For instance, during the Lovastatin biosynthesis LovC only reduces tetra-, penta- and heptaketide intermediates (Ames *et al.*, 2012). Another example is the *trans*-acting ER FSL5 in *Fusarielin* BGC that acts on $C_{10}=C_{11}$ or $C_{12}=C_{13}$ to help in the synthesis of the corresponding polyketide (Droce *et al.*, 2016).

II.7 THIOESTER DOMAIN (TE)

Termination of polyketide biosynthesis involves the TE domain, which produces macrolactones via intramolecular cyclization or linear polyketides by hydrolysis (Keatinge-Clay, 2012). In both events, an acyl-TE intermediate is formed through the transfer of the polyketide chain from the last ACP to the active serine on TE domain (Jenner, 2016). In the hydrolysis mechanism, the active site of the TE domain (SxDxH) causes a nucleophilic attack on the carbonyl of the thioester, which occurs when the conserved Asp residue is stabilized to receive a proton from the serine residue present in this motif. Stabilization is carried out when the histidine acts as catalytic base. During macrolactone formation, a similar AT-mechanism is involved, although during the second half of the reaction the acyl component of the acyl-enzyme intermediate gives the nucleophile needed during the reaction. This occurs when a secondary alcohol/amine is activated by the catalytic histidine to perform the ester attack and release the cyclic product (Keatinge-Clay, 2012; Bruegger *et al.*, 2014). The TE domain consists of around 270 residues and has an α,β -hydrolase fold (acting in *cis* or *trans*) or the *hot-dog* fold. Typically, the TE domain in modular type I PKS systems is dimeric and the iterative type I PKS is monomeric (Jenner, 2016). Usually, TE domains are localized at the C-terminus of the last module. This arrangement is

also the case for special TE domains such as the TE/CLC (Claisen-like cyclase) type, which is implicated in the formation of a new ring system during the biosynthesis of aflatoxin and naphthopyrone (Keller *et al.*, 2005; Nakamura *et al.*, 2015).

II.8 POLYKETIDE SYNTHASE CLASSIFICATION

According to their protein architecture and mode of action, PKS enzymes are classified into three types.

Type I PKSs are mainly found in bacteria and fungi. These multidomain proteins can be further subdivided in two categories: *modular* and *iterative* (Nair *et al.*, 2012). *Modular type I PKSs* or *non-iterative PKSs* are unique for bacteria and are characterized by presenting a sequence (or set) of modules, each constituted with set of specific catalytic domains. In consequence, the number of precursors fused in the polyketide is equivalent with the number of modules that are present (Chan *et al.*, 2009). The 6-deoxyerythronolide B synthase (DEBS), which catalyzes the synthesis of the aglycone core in erythromycin A (Figure 2A), is a representative example of this category since during the chain extension each cycle is performed by a different module (Kellenberger *et al.*, 2008; Stevens *et al.*, 2013). In contrast, *iterative type I PKSs* use the same catalytic core domains as modular type I PKSs, but the catalytic reaction is repeated to yield the complete polyketide backbone. A representative example of this type is LovB, that together with LovC (a enoyl reductase) catalyzes around 35 reactions to produce dihydromonacolin L, an intermediate in lovastatin biosynthesis (Campbell and Vederas, 2010).

Type II PKSs are unique for bacteria and use a similar iterative mechanism as observed in *iterative type I PKSs*. However, the different catalytic domains are encoded by independent genes. In general, they often constitute a “minimal PKS”, that comprises two KS units (KS α and KS β) and an ACP protein that holds the growing PKS chain (Figure 2D). The KS β domain defines the length of the polyketide chain. The folding pattern of the poly- β -keto intermediates is determined by optional PKS units such as aromatases, ketoreductases and cyclases.

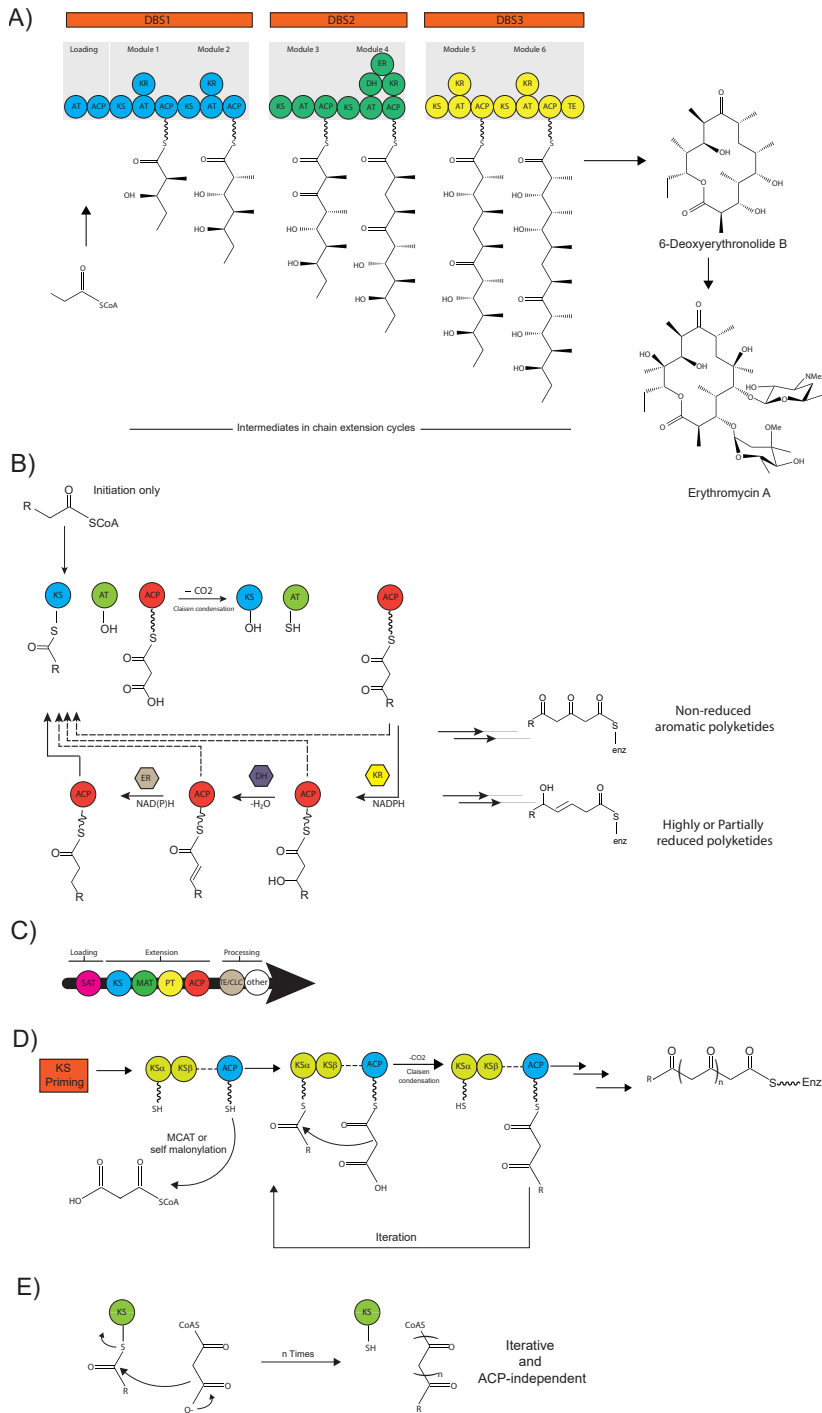
Other tailoring modifications are performed by oxygenases, methyl and glycosyl transferases. Known metabolites synthesized by type II PKSs are tetracyclines, anthracyclines and aureolic acids (Hertweck *et al.*, 2007; Jenner, 2016).

Type III PKSs have originally been discovered in plants, but are also present in bacteria and fungi. They consist of a single KS domain that catalyzes a defined number of elongations (Figure 2E), usually generating small phenols or naphthol rings. The enzyme transfers the acyl group from the CoA to the active site histidine, which is a highly conserved residue. However, the amino acid sequence of the His motif is not similar to those found in KS domains of type I and II PKS enzymes (Shen, 2003; Chan *et al.*, 2009; Bruegger *et al.*, 2014; Jenner, 2016). Importantly, independent of the mechanistic or structural differences, all the polyketides synthesized by PKS enzymes follow the same decarboxylative condensation mechanism of the acyl CoA precursors. However, these precursors should prior be activated by the ACP domain, in the case of the type I and II PKS enzymes, whereas type III PKS enzymes act independently of ACP domain (Shen, 2003; Weissman, 2009). Acridones, pyrenes, chalcones are some examples of the compounds produced by type III PKS enzymes (Yu *et al.*, 2012).

II.8.1 FUNGAL ITERATIVE PKSS

Like type I PKS enzymes, fungal PKSs have a modular organization and the consecutive domains act in sequential order during the synthesis of the complete polyketide. They are equipped with basic structural domains typically found in PKS enzymes (ACP-KS-AT domains) but may also contain optional units (KR,DH,ER,TE domains). Depending

Figure 2. Polyketide processing elements. A) Modular Type I PKS. Erythromycin biosynthesis. B) Elongation and reduction process for Iterative Type I PKS (fungal). C) NR-PKS domain structure of Iterative Type I PKS. D) Type II PKS: iterative mechanism. MCAT (malonyl-CoA:ACP transferase). E) Type III PKS: iterative and ACP- independent. For details see the text. Adapted from (Hertweck *et al.*, 2007; Weissman, 2009; Campbell and Vederas, 2010; Cox and Simpson, 2010; Crawford and Townsend, 2010; Stevens *et al.*, 2013).



on the presence or absence of reducing domains, these enzymes can be divided into *highly reducing* (HR), *non-reducing* (NR) and *partially reducing* (PR) PKS (Figure 2B–C).

Highly reducing PKS (HR-PKS) produces the linear or cyclic scaffold of some compounds as fumonisins, T-toxines, solanapyrone E, squalestatin, lovastatin (Chiang *et al.*, 2014; Roberts *et al.*, 2017). Usually, they start with a KS domain, followed by an AT, DH and C-Met domain, although this latter not always follows the DH domain. The ER domain is an optional unit in HR-PKS enzymes, but when the ER is missing, the corresponding region is filled with a polypeptide domain with unknown function. Furthermore, these enzymes do not contain a product template domain (PT) or N-terminal SAT domain, whereas these special domains are present in NR-PKS enzymes (Cox and Simpson, 2010).

Partially reducing PKS (PR-PKS). Structurally, these enzymes have a domain architecture that is similar to the mammalian FAS: a N-terminal KS-domain followed by a AT-, DH-, and “core”-KR-ACP domain. These enzymes lack an ER domain (L. Wang *et al.*, 2015), and also do not have a TE domain, which suggests an alternative mechanism of product release than hydrolysis. PR-PKS enzymes produce small aromatic molecules such as 6-methylsalicylic acid (MSA), but in most cases the chemical product is unknown (Cox and Simpson, 2009, 2010; Kage *et al.*, 2015).

Non-reducing PKS (NR-PKS) are involved in the biosynthesis of aromatic polyketides, which contain conjugate aromatic rings. Typically, these enzymes consist of six catalytic domains that are covalently tethered and arranged in four components: *loading* (SAT), *chain extension* (KS-MAT-PT-ACP), *cyclisation and processing components* (TE-CLC). The ACP transacylase (SAT) domain acts as starter unit that loads the acyl carrier protein whereupon chain extension is mediated for KS and AT domain. During this process, the malonyl-CoA:ACP transacylase (MAT) domain transfers the extension units from malonyl-CoA to the ACP, while the product template (PT) domain stabilizes the reactive poly- β -keto intermediates. The processing component acts after the initial assembly when the cyclized or polyketide intermediate is still attached to the ACP. Final cyclization and release is catalyzed by the

TE/Claissen cyclase (CLC) domain. Aflatoxin, orsellenic acid, griseofulvin, zearalenone and bikaverin are some example of products produced by NR-PKS. (Cox and Simpson, 2010; Crawford and Townsend, 2010; Bruegger *et al.*, 2013; Chiang *et al.*, 2014).

III. POLYKETIDES OF *PENICILLIUM CHRYSOGENUM*

Table 1 lists all PKS genes of *P. chrysogenum* and insofar known the associated products.

Table 1. Polyketide synthases in *P. chrysogenum* and (insofar known) their associated products. ks, ketosynthase; at, acyltransferase; dh, dehydratase; mt, methyltransferase; er, enoyl reductase; kr, ketoreductase; acp, acyl carrier protein; te/red, thioester reductase. (*) Point mutations present in *pks* genes of industrial *P. chrysogenum* strains subject to CSI program. Modified from Samol *et al.*, 2016.

| Gene ID | Gene Name | Protein | Domain organization | Product/Pathway |
|------------|-----------------------|----------------------|-----------------------|------------------|
| Pc12g05590 | <i>pks1</i> | - | ks-at-dh-mt-kr-acp | - |
| Pc13g04470 | <i>pks2*</i> | - | ks-at-dh-mt-er-kr-acp | - |
| Pc13g08690 | <i>pks3</i> | - | ks-at-dh-mt-er-kr-acp | - |
| Pc16g00370 | <i>PcYanA(pks4)</i> | 6-MSA synthase | ks-at-kr-acp | 6-MSA/Yanuthones |
| Pc16g03800 | <i>pks5</i> | - | ks-at-dh-er-kr-acp | - |
| Pc16g04890 | <i>pks6</i> | - | ks-at-dh-mt-er-kr-acp | - |
| Pc16g11480 | <i>pks7*</i> | - | ks-at-dh-mt-er-kr-acp | - |
| Pc21g00960 | <i>pks8*</i> | - | ks-at-dh-mt-er-kr-acp | - |
| Pc21g03930 | <i>pks9</i> | - | ks-at-dh-mt-er-kr-acp | - |
| Pc21g03990 | <i>pks10</i> | - | ks-at-dh-er-kr-acp | - |
| Pc21g04840 | <i>pks11</i> | - | ks-at-dh-er-kr-acp | - |
| Pc21g05070 | <i>SorB(pks12)*</i> | Sorbicillin synthase | ks-at-acp-mt-te/red | Sorbicillinoids |
| Pc21g05080 | <i>SorA(pks13)*</i> | Sorbicillin synthase | ks-at-dh-mt-er-kr-acp | Sorbicillinoids |
| Pc21g12440 | <i>pks14</i> | - | ks-at-dh-er-kr-acp | - |
| Pc21g12450 | <i>pks15*</i> | - | ks-at-acp-te | - |
| Pc21g15160 | <i>pks16</i> | - | ks-at-dh-mt-er-kr-acp | - |
| Pc21g16000 | <i>PcAlb1(pks17)*</i> | YWA1 synthase | ks-at-acp-acp-te | YWA1/DHN-Melanin |
| Pc22g08170 | <i>PcPatK(pks18)</i> | 6-MSA synthase | ks-at-kr-acp | 6-MSA/Patuline |
| Pc22g22850 | <i>AdrD(pks19)</i> | DMOA synthase | ks-at-acp-mt-te/red | DMOA/Andrastin A |
| Pc22g23750 | <i>pks20</i> | - | ks-at-dh-mt-er-kr-acp | - |

III.1 SORBICILLINOIDS

Sorbicillinoids are yellow pigments that already during the earlier stages of the CSI programme were eliminated from *P. chrysogenum*, since they interfered as a colored contamination during antibiotic production. However, in recent years the interest in this group of molecules has revived due to the pharmacological activities of some sorbicillinoids. These compounds belong to a family of hexaketide metabolites that were originally isolated from *P. notatum* in 1948. More than 90 molecules have been detected in a range of fungi such as *Trichoderma*, *Emericella*, *Acremonium*, *Verticillium* and some marine ascomycetes (Harned and Volp, 2011; Salo *et al.*, 2015; Meng *et al.*, 2016). Structurally, the sorbicillinoids are highly oxygenated molecules with bicyclic and tricyclic ring skeletons (structures). The C1'-C6' sorbyl side chain is a typical feature present in the sorbicillinoids. These structures are produced by oxidative dearomatization, dimerization/tridimerization of sorbicillin and modifications introduced by rearrangement reactions (Bringmann *et al.*, 2005; Meng *et al.*, 2016). Therefore, these molecules can be grouped as monomeric sorbicillinoids, bisorbicillinoids, trisorbicillinoids and hybrid sorbicillinoids (Meng *et al.*, 2016).

Sorbicillinol is the most common and reactive form of the monomeric sorbicillinoids, and is a key pathway intermediate (Fahad, 2014). For instance, sorbicillinol can be converted to bisvertinolone through a nucleophilic attack from the carbanion (formed in oxosorbicillinol) to C-1 in sorbicillinol. Additionally, sorbicillinol is also the precursor of two bisorbicillinoids: bisorbicillinol and trichodimerol (N. Abe *et al.*, 2002). These compounds respectively exhibit an antioxidant effect and indirectly inhibit the tumor necrosis factor- α (TNF- α) (Sugaya *et al.*, 2008). Overall, monomeric and dimeric sorbicillinoids have a radical-scavenging activity, albeit some bisorbicillinoids show antimicrobial and anticancer activities. Recently, some sorbicillinoids have been described that act as potential inhibitors of target proteins associated with diabetes and HIV (Du *et al.*, 2009; Harned and Volp, 2011).

Regarding the sorbicillinoids biosynthesis, isotope labelling studies showed that the formation of the hexaketide structure of sorbicillinol is performed by a Claisen-type reaction (Sugaya *et al.*, 2008; Harned and Volp, 2011). Further sorbicillinoid dimers are assembled by Diels-Alder and Michael-type reactions (Maskey *et al.*, 2005; Du

et al., 2009). Likewise, in *P. chrysogenum* E01-10/3, two polyketide synthases were suggested to be involved in the synthesis of sorbicil-lactone A/B (Avramović, 2011). In *P. chrysogenum*, it was demonstrated that SorA, a highly reducing polyketide synthase, is essential for sorbicillinoid biosynthesis (Salo *et al.*, 2016). Interestingly, this PKS gene belongs to a gene cluster that contains another PKS gene and this gene cluster is also found in some *Hypocreales*. While in *Colletotrichum graminicola* only the two polyketide synthases genes are present. *P. chrysogenum* contains a cluster of seven genes, which includes the two polyketide synthases (*sorA*, Pc21g05080; *sorB*, Pc21g05070), two transcriptional regulators, a transporter of the major facilitator superfamily (MFS), one monooxygenase and one oxidase. Despite the fact that this cluster is also present in industrial improved strains of *P. chrysogenum*, these strains do not produce sorbicillinoids due to a point mutation in the ketosynthase domain of SorA (Druzhinina *et al.*, 2016; Salo *et al.*, 2016). Recently, the biosynthetic pathway and regulatory mechanism have been resolved, wherein sorbicillinoids act as autoinducers (Guzmán-Chávez *et al.*, 2017).

III. 2 ω -HYDROXYEMODIN

ω -Hydroxyemodin (OHM), also called citreorsein, is a bright-yellow pigment isolated of *P. cyclopium*. This compound is a derivative of emodin, which is a molecule common for terrestrial *Penicillium* strains (Fouillaud *et al.*, 2016; Samol *et al.*, 2016). Recently, It was demonstrated that OHM inhibits quorum sensing involved in the production of virulence factors in *Staphylococcus aureus* by direct binding of OHM to the regulatory protein. Interestingly, ω -hydroxyemodin was isolated from solid cultures of *P. restrictum* (Daly *et al.*, 2015). Likewise, OHM reduces the production of proinflammatory cytokines in mast cells, which play an important role in the inflammatory and hypersensitivity response during the immune response (Lu *et al.*, 2012). It has been suggested that *P. chrysogenum* is also able to produce OHM, since this compound was detected in cultures of *P. citreorseum*, a strain that was later identified as *P. chrysogenum* (Rajagopalan and Seshadri, 1956; Houbraken *et al.*, 2011). OHM is an anthraquinone, that like aflatoxins and xanthenes, constitutes an octaketide structure whose

biosynthesis seems to occur through the acetate-malonate pathway involving non-reducing polyketide synthases (Brase *et al.*, 2009; Fouillaud *et al.*, 2016). In the case of ω -hydroxyemodin, the genes involved in its synthesis have not been identified yet.

III. 3 6-METHYLSALYCILIC ACID (6-MSA)

6-MSA is one of the smallest polyketides, whose structure contains a cyclized eight carbon chain. This molecule is produced by 6-methyl salicylic acid synthase (6-MSA synthase), a partial reducing PKS (Wattanachaisaereekul *et al.*, 2007; Gallo *et al.*, 2013). This enzyme contains a KS, MAT, DH, KR and ACP domain, which are responsible to assemble three molecules of malonate and one molecule of acetate into 6-MSA by a sequence of reactions in the following order: two condensation reactions, a reduction, a dehydration, a third condensation, and cyclisation reaction and the release of the final molecule (Staunton and Weissman, 2001). *P. chrysogenum* contains two PKS genes that encode for 6-MSA synthases. These genes belong to different BGCs involved in the biosynthesis of the mycotoxin patulin (Pc22g08170) and the antibiotic yanuthone D (Pc16g00370). The synthesis of these molecules involves three common steps. Despite the fact that these two BGCs are highly conserved in the *Penicillium* genus, some species only harbor one cluster, while others such as *P. chrysogenum* only contains a full version of one cluster (yanuthone D BGC), while the second cluster (patulin BGC) is incomplete (Nielsen *et al.*, 2017). Indeed the absence of the gene encoding for isoeopoxidon dehydrogenase in *P. chrysogenum* agrees with the fact that this fungi does not produce patuline (Samol *et al.*, 2016). However, under laboratory conditions yanuthone D is also not detected in this fungus (Salo, 2016).

III. 4 YWA1

The heptaketide naphthopyrone YWA1 is a pigment present in spores which provides rigidity and impermeability to the cell wall (Crawford and Townsend, 2010). In *Aspergillus nidulans*, YWA1 is produced by the non-reducing polyketide synthase WAS. This PKS catalyzes the

cyclization and aromatization of the first ring (Cox, 2007). Interestingly, the TE-CLC domain in other NR-PKSs is responsible for the synthesis of compounds such as norsolorinic acid, anthrone and bikaverin. Indeed, when the carboxy-terminal TE domain is removed from WAS, citreoisocoumarins is produced instead of YWA1 (Crawford and Townsend, 2010). In *A. fumigatus*, the protein Alb1P is an orthologue protein of WAS. This corresponding gene belongs to the melanin BGC that is expressed during the conidiation process. During the biosynthesis of melanin, the product formed by Alb1P (YWA1) is converted to 1,3,6,8 tetrahydroxynaphthalene (THN) by Ayl1p through a retro Claisen mechanism. After the reduction of THN by ARP2, a dehydration step is performed by the enzymes encoded by the *arp1* and *abr1* genes that encode scytalone and vermellone dehydratases, respectively. The final polymerization is catalysed by a laccase (encoded by the *abr2* gene) that converts 1,8-DHN into DHN-melanin (Pihet *et al.*, 2009). In *P. chrysogenum*, the orthologue genes involved in the melanin biosynthetic pathway are: *pks17* (Pc21g16000), *ayg1* (Pc21g16440), *abr1* (Pc21g16380), *apr1* (Pc21g16420), *arp2* (Pc21g16430), and *abr2* (Pc22g08420). However, these genes are only partially clustered in the genome (Salo, 2016).

III. 5 ANDRASTIN A

Andrastin A is a metabolite produced by several species from the *Penicillium* genus. It is a potential antitumor agent and functions as a farnesyl transferase inhibitor (Overy *et al.*, 2005; Matsuda *et al.*, 2013). Recently, the Andrastin A biosynthetic gene cluster has been described in *P. roqueforti* and *P. chrysogenum*. This BGC consists of eleven genes designated *adrA-J* (Pc22g22820 to Pc22g2292), that encode for a cytochrome P450, hypothetical protein, ABC transporter, polyketide synthetase, ketoreductase, short chain dehydrogenase, prenyltransferase, FAD-dependent monooxygenase, terpene cyclase, acetyltransferase and methyltransferase, respectively (Matsuda *et al.*, 2013; Rojas-Aedo *et al.*, 2017). *AdrD* (PKS 19) is a PKS that catalyse the first reaction to produce a tetraketide (3,5-dimethylorsellinic acid, DMOA) from acetyl CoA, malonyl CoA and S-adenosylmethionine. The following steps in the Andrastin A biosynthetic pathway are performed by *AdrG*, *AdrK*

and AdrH to produce the epoxyfarnesyl-DMOA methyl precursor. Using a heterologous co-expression approach in *Aspergillus oryzae*, the pathway has been resolved, although the role of AdrB and AdrC is unknown (Rojas-Aedo *et al.*, 2017). Interestingly, *P. chrysogenum* strains that were subject to CSI are not able to produce Andrastin A or any related compound.

IV. STRATEGIES TO ACTIVATE SILENT GENE CLUSTERS

Natural products represent a broad range of molecules produced by animals, plants and microorganisms. These molecules may display different biological activities (e.g. antiviral, antimicrobial, anti-tumor, immunosuppressive agents) and it is estimated that the majority of these compounds are derived from filamentous fungal sources and from filamentous bacteria belonging to the genus *Streptomyces*. With respect to antibiotics, most of the chemical scaffolds used today were discovered during the golden age of antibiotics discovery (1940s–1960s). This was followed by four decades during which hardly any new scaffolds from a natural source were developed (Reen *et al.*, 2015; Smanski *et al.*, 2016; Okada and Seyedsayamdost, 2017). However, there is also a current understanding that only a small fraction of the potential possible molecules has been discovered to this date. This follows from genomic studies revealing large numbers of uncharacterized BCGs, while many of these gene clusters are not expressed (silent or sleeping gene clusters) under laboratory conditions (Brakhage and Schroeckh, 2011). Furthermore, metagenomics studies indicate that the majority of microbes present in the environment have not been cultured and characterized. Thus, there are many challenges that need to be overcome in order to harness the natural diversity of natural products, to cultivate potential strains under laboratory conditions and activating the BGCs for expression. To achieve the latter, two main approaches are used in recent years: *manipulation of cultivation conditions* and *genetic interference*.

IV.1 MANIPULATION OF CULTIVATION CONDITIONS

Under natural conditions, fungi face a variety of biotic and abiotic conditions to survive. The cellular response to the environment involves complex regulatory networks that respond to stimuli such as light, pH, availability of carbon and nitrogen sources, reactive oxygen species, thermal stress, and interspecies-crosstalk (Brakhage, 2012; Reen *et al.*, 2015).

OSMAC (*one strain many compounds*) approach. This strategy is derived from the observation that changes in the metabolic output of microorganisms can be achieved by alternating the medium composition and other cultivation parameters. It is well known that glucose, ammonium or phosphate at high concentrations act as repressors of secondary metabolism, whereas iron starvation and nitrogen limitation can stimulate secondary metabolite production. The latter is for instance exploited for the production of terrain by *A. terreus* (Bode *et al.*, 2002; Brakhage and Schroeckh, 2011; Gressler *et al.*, 2015). This strategy can readily be implemented using high-throughput methods, where an array of culture conditions can be screened for new metabolite profiles (Spraker and Keller, 2014). In combination with bioinformatics tools, this strategy can be a powerful tool to investigate the production of new molecules, as exemplified by the discovery of aspoquinolones A–D in *A. nidulans* (Scherlach and Hertweck, 2006). However, despite the fact that the OSMAC approach has led to the discovery of increased numbers of new molecules with antimicrobial activity, some chemical and physical conditions are still missing under the laboratory tested conditions as often the activation concerns a limited number of BGCs (Chiang *et al.*, 2009).

Interspecies-crosstalk. The production of secondary metabolites is a natural strategy that microorganisms have developed to cope with specific environmental conditions and challenges. They serve as intermediary agents to establish a symbiotic association between species or as a weapon against other organism to compete for nutrients and space. These conditions, that are not present in axenic cultures, boost the production of molecules that are constitutively present and/or that are cryptic and normally are not synthesized due to silencing of the respective BGCs (Demain and Fang, 2000; Marmann *et al.*, 2014). The strategy

in which different organisms are cultivated together is called “co-culture”, which has been successful in several cases yielding new metabolites. *A. fumigatus* produces fumiformamide when co-cultivated with *Streptomyces peucetius*, while co-cultivation of this fungi with *Streptomyces rapamycinicus* results in the production of fumicyclines A and B, two novel polyketides with antibacterial activity, are examples of the use of this strategy (Netzker *et al.*, 2015; Adnani *et al.*, 2017). Interestingly, the association of two marine organisms, *Emericella sp* and *Salinispora arenicola*, results in the biosynthesis of emericellamides A and B which are equipped with antibacterial activity (Oh *et al.*, 2007). Also, the interactions between fungi and insects result in the production of volatile secondary metabolites (Rohlf and Churchill, 2011).

IV.2 GENETIC INTERFERENCE

Another mechanism to stimulate the expression of silent BGCs is by genetic interference, for instance by direct manipulation of the regulatory network related to BGCs expression. The regulation of BGCs is effected at many levels, through specific (or local) and global regulators up to epigenetic regulation involving the modification of the chromatin landscape (Lim *et al.*, 2012; Spraker and Keller, 2014).

IV.2.1 MANIPULATION OF GLOBAL REGULATORS

Pleiotropic transcriptional regulators or global regulators are proteins that respond to environmental signals such as pH, temperature, and N- and C-sources. They provide the link between the production of secondary metabolites and external cues. In fungi, these proteins control the regulation of BGCs that do not contain other regulatory factors. About half of the known clusters do not encode a local and specific regulator. Additionally, global regulators also act over genes that do not belong to secondary metabolism (Brakhage, 2012; Rutledge and Challis, 2015; Fischer *et al.*, 2016). Global regulators that have been reported as key players in the biosynthesis of secondary metabolites are featured below.

Velvet complex. This heterotrimeric complex is a conserved regulator present in most of the fungi, except yeast. It consists of at least three proteins: VeA, VelB and LaeA. Likewise, this complex provides a link between sexual development and secondary metabolism through light regulation (Yin and Keller, 2011; Deepika *et al.*, 2016), since light has an inhibitory effect on VeA expression. The formation of the velvet complex takes place in the nucleus, where the complex VeA-VelB via the α -importin KapA meets the methyltransferase LaeA. It has been hypothesized that the velvet complex acts as a transcriptional factor as it contains a DNA binding fold that resembles the corresponding region of the NF- κ B transcription factor of mammals (Sarıkaya-Bayram *et al.*, 2015). The role of the velvet complex in secondary metabolism mostly follows from the control that the LaeA protein executes on several BGCs in filamentous fungi. LaeA (loss of aflR expression-A) was identified in 2004 as a global regulator in *Aspergillus*. Deletion of this gene results in the repression of many BGC, such as the one responsible for the production of penicillin, lovastatin and sterigmatocystin. Overexpression of LaeA causes an opposite phenotype. Interestingly, LaeA is negatively regulated by AflR (Zn₂Cy₆ transcriptional factor) in a feed loop mechanism (Bok and Keller, 2004). It has been hypothesized that LaeA acts at different levels, i.e., as a methyltransferase, epigenetically and as a direct member of the velvet complex. Structurally, LaeA has a S-adenosyl methionine (SAM)-binding site with a novel S-methylmethionine auto-methylation activity, although this activity does not seem to be essential for its function. LaeA is not a DNA-binding protein, but it does affect chromatin modifications. In an *A. nidulans* Δ laeA strain, high levels of the heterochromatin protein 1 (HepA) are detected and an increase in trimethylation of the H3K9 in the sterigmatocystin cluster. When LaeA is present, the levels of HepA, ClrD (H3K9 methyltransferase) and H3K9me3 decrease while the sterigmatocystin levels are raised. The heterochromatic marks stay until the sterigmatocystin cluster is activated, and apparently LaeA influences the offset of these marks in this particular cluster (Reyes-Dominguez *et al.*, 2010; Brakhage, 2012; Jain and Keller, 2013; Sarıkaya-Bayram *et al.*, 2015; Bok and Keller, 2016). Orthologues of LaeA have been discovered in many other filamentous fungi as *Penicillium*, *Fusarium*, *Trichoderma*, *Monascus spp* and LaeA exhibits positive and negative effects on the synthesis of natural products. For instance,

LaeA1 of *Fusarium fujikuroi* positively regulates the production of fusarin C, fumonisins and gibberellins, and represses bikaverin biosynthesis. In *P. chrysogenum*, LaeA controls the biosynthesis of penicillin, pigmentation and sporulation. In *Trichoderma reesei*, Lae1 positively modulates the expression of cellulases, xylanases, β -glucosidases. Interestingly the stimulation of these genes was not directly influenced by the methylation of H3K4 or H3K9 (Wiemann *et al.*, 2010; Yin and Keller, 2011; Lim *et al.*, 2012; Seiboth *et al.*, 2012; Jain and Keller, 2013).

LaeA is not the only member of the velvet complex that has influence on the regulation of secondary metabolite production. VeA of *A. parasiticus* is necessary for the expression of two transcriptional factors of the aflatoxin cluster (AflR and AflJ), which regulate the pathway. In *Aspergillus fumigatus*, veA regulates twelve BGCs (Dhingra *et al.*, 2013). This study also revealed that veA modulates the biosynthesis of fumagillin via the regulation of fumR, a transcriptional factor of the fumagillin cluster, which in turn is also regulated by LaeA. Similarly, a transcriptome analysis in *A. flavus* revealed that 28 of 56 BGCs are dependent on veA, in particular the aflavarin cluster which is differentially expressed. Likewise, orthologues of veA are also present in other fungi such as in *P. chrysogenum*, *Fusarium oxysporum*, *Botrytis cinerea*, *Fusarium verticillioides* (Yin and Keller, 2011; Dhingra *et al.*, 2013; Jain and Keller, 2013; Cary *et al.*, 2015). Despite the clear interaction between veA and LaeA in the velvet complex and its influence on secondary metabolism, it is thought that veA may be acting as molecular scaffold of the velvet complex, since it interacts with other three methyl transferases (LaeA-like methyltransferase F (LlmF), velvet interacting protein C (VipC), and VipC associated protein B (VapB)). This suggests that veA functions in a supercomplex or in dynamic network control. Taken together, modulation of the velvet complex is a useful tool to activate BCGs (Sarıkaya-Bayram *et al.*, 2015).

bZIP transcription factors are highly conserved in the eukaryote domain. The dimeric basic leucine zipper (bZIP) transcriptional factors play an important role in the cellular responses to the environment. Regarding the structure, they contain a conserved leucine zipper domain and a basic region, which controls the dimerization of the protein and establishes sequence-specific DNA-binding, respectively. Once dimeric, bZIPs target palindromic DNA sequences by two mechanisms: redox

and phosphorylation (Amoutzias *et al.*, 2006; Knox and Keller, 2015). In fungi, bZIP proteins have been implicated in multiple metabolic processes, such as in the regulation of development, morphology and in stress responses. Several orthologues of the Yap family bZIPs, which were first described in yeast, have been characterized in *Aspergillus spp* (AtfA, NapA, Afyap1, Aoyap1, and Apyap1) and these regulators have recently been associated with the production of secondary metabolites in filamentous fungi. In *A. nidulans*, overexpression of *RsmA* (restorer of the secondary metabolism A, Yap-like bZIP) has a compensatory effect on secondary metabolism in a strain in which *LaeA* and *veA* are missing. However, these transcription factors also display negative regulation. For instance, an increase in the biosynthesis of aflatoxin and chratoxin has been observed when *yap1* is deleted in *A. parasiticus* and *A. ochraceus* (Yin *et al.*, 2013; Knox and Keller, 2015; X. Wang *et al.*, 2015). *MeaB* is another bZIP transcriptional factor that was discovered in *A. nidulans*. Its function is associated in nitrogen regulation and has a negative effect on the biosynthesis of aflatoxin in *A. flavus* and bikaverin production in *F. fujikuroi*. (Wagner *et al.*, 2010; Amaike *et al.*, 2013).

Other global regulators. *AreA* is a highly conserved transcriptional factor in fungi that belongs to the GATA family and it is characterized by Cys₂Hys₂ zinc finger DNA binding domains. Likewise, it is involved in the repression of nitrogen metabolism when ammonium or glutamine are present. Recently, this transcription factor and its orthologues have been shown to influence secondary metabolism. For instance, *areA* deletion strains of *Fusarium verticillioides* are not able to produce fumonisins on mature maize kernels. In *Acremonium chrysogenum*, the deletion of *areA* resulted in the reduction of cephalosporin because of a reduced expression of the enzymes involved in cephalosporin biosynthesis. Additionally, *AreA* is a positive regulator of the production of gibberellins, trichothecene deoxynivalenol (DON), fusarielin H, beauvericin and zearalenone (Li *et al.*, 2013; Tudzynski, 2014; Knox and Keller, 2015). The carbon catalytic repressor *CreA* also influences secondary metabolism. *CreA* is a Cys₂His₂ zinc finger transcription factor that is involved in the repression of genes associated with the use of carbon sources other than glucose (Knox and Keller, 2015). This transcription factor acts by direct competition with activator proteins

for specific binding sites (5'-SYGGRG-3') and by direct interaction with activators (Janus *et al.*, 2008). In *P. chrysogenum* CreA represses penicillin biosynthesis and causes a reduced expression of the *pcbAB* gene that encodes nonribosomal peptide synthetase involved in this pathway. Mutations in the putative CreA binding site in the *pcbAB* promoter result in enhanced enzyme expression when cells are grown in the presence of glucose (Cepeda-García *et al.*, 2014). In contrast, mutations in the CreA binding sites of the *ipnA* promoter (*pcbC* in other species) of *A. nidulans* revealed that in this organism repression of penicillin biosynthesis by glucose is independent of CreA (Knox and Keller, 2015). CreA has been implicated in the variable metabolite profiles when fungi are grown in the presence of different carbon sources (Yu and Keller, 2005). Recently, the xylanase promoter binding protein (Xpp1) of *Trichoderma reesei* was used as a reporter to fulfil a dual role in the regulation of primary and secondary metabolism. Xpp1 is an activator of primary metabolism, while its deletion boosts the production of secondary metabolites, including sorbicillinoids (Derntl *et al.*, 2017). Another Cis_2His_2 zinc finger transcription factor conserved in fungi is PacC, which is involved in pH dependent regulation. Deletion of the orthologue of this gene (BbpacC) in *Beauveria bassiana* resulted in a loss of dipicolinic acid (insecticide compound) and oxalic acid production, compounds that reduce the pH of the medium. However, also production of a yellow pigment was noted. When *A. nidulans* is grown at alkaline pH, PacC modulates the expression of the *acvA* (*pcbAB*) and *ipnA* of the penicillin BGC, while it acts negatively on the expression of the sterigmatocystin BGC (Deepika *et al.*, 2016; Luo *et al.*, 2017). In filamentous fungi, another global regulatory element is the CCAAT-binding complex (CBC). This complex consists of three proteins (HapB, HapC and HapE) that respond to redox stimuli and an additional unit HapX, a bZIP protein that interacts with the complex for modulating the iron levels. In *A. nidulans* this complex binds to CAATT motifs, which are present in the penicillin BGC stimulating the expression of the *ipnA* and *aatA* (*penDE*) genes (Bayram and Braus, 2012; Brakhage, 2012). Whereas in *F. verticillioides* the orthologue core of this complex (FvHAP2, FvHAP3, and FvHAP5) is deleted, cells show an altered hyphal morphology, reduction of growth, reduced pathogenesis and a deregulation of secondary metabolism (Ridenour and Bluhm, 2014).

IV.2.2 MANIPULATION OF SPECIFIC REGULATORS

In addition to the global regulators, BGCs can be also modulated by specific regulatory elements, which most of the times are encoded by genes that are part of the same cluster that they regulate. In some cases, such regulators also influence the expression of other BGCs. It is estimated that around 60% of the fungal BGCs contain a putative regulator. With PKS containing BGCs this even amounts to 90% and mostly concerns a regulator that belongs to the Zn_2Cys_6 binuclear cluster domain family. With NRPS containing BGCs, the putative transcription factors are more diverse. The Zn_2Cys_6 family of transcription factors contain a DNA binding domain (DBD) that has two zinc atoms coordinated by six cysteines. There are three sub regions: a linker, a zinc finger and a dimerization domain. Additional to DBD, these proteins contain two further functional domains, the acidic region and the regulatory domain. These transcription factors can act as monomers, heterodimers and homodimers. They recognize single or multiple trinucleotide sequences, commonly CCG triples, in a symmetric or asymmetrical format. The affinity of the DBD for a given DNA stretch is also determined by the nucleotides surrounding this triplet. The transcriptional activity of these proteins is regulated by phosphorylation, exposing the activation and DNA binding domains for DNA binding (MacPherson *et al.*, 2006; Brakhage, 2012). Some of these regulators have been shown to control the expression of BGCs. For instance, in *Fusarium verticillioides* the disruption of *FUM21* gene, that encodes a Zn_2Cys_6 protein, reduces fumonisin production as a result of a downregulation of the BGC (Brown *et al.*, 2007). Interestingly, fumonisin production is also regulated by another Zn_2Cys_6 protein that is encoded by a gene located outside of the fumonisin cluster (Flaherty and Woloshuk, 2004). *Micr* is another example of a positive regulator that controls compactin production in *P. citrum* (Y. Abe *et al.*, 2002). *AflR* is a Zn_2Cys_6 protein that regulates the biosynthesis of aflatoxin/sterigmatocystin through binding to a palindromic sequence (5'-TCG(N₅)GCA) that is found in most of the promoters of this BGC, albeit a second binding sequence has been reported that is associated with the autoregulation mechanism of the expression of *AflR*. The disruption of *AflR* abolishes the production of aflatoxin/sterigmatocystin. Likewise, some BGCs encode multiple regulatory proteins. Next to the *aflR* gene in the aflatoxin cluster resides

the *aflS* (formerly *aflJ*) gene. The corresponding transcription factor binds to AflR to enhance the transcription of early and mid-biosynthetic genes in the aflatoxin pathway (Georgianna and Payne, 2009; Yin and Keller, 2011). In *P. chrysogenum* and *Trichoderma reesei*, the sorbicillin BGC is regulated by two transcriptional factors through a coordinated action. Also, regulation of BGCs via crosstalk has been observed in filamentous fungi. For instance, the alcohol dehydrogenase promoter has been used to induce the expression of putative pathway-specific regulatory gene (*scpR*) in *A. nidulans*, which controls the expression of two pathway associated NRPS genes (*inpA* and *inpB*). Surprisingly, two PKS genes (*afoE* and *afoG*) and one transcriptional activator (*afoA*) belonging to the asperfuranone BCG are also upregulated by ScpR, allowing the production of asperfuranone (Bergmann *et al.*, 2010). For some regulators, no clear phenotype is observed. For instance deletion of the *chyR* gene of the chrysogine BGC in *P. chrysogenum*, has no effect on the expression of the corresponding BGC (Viggiano *et al.*, 2017).

IV.2.3 CHROMATIN-MEDIATED REGULATION.

In the cell, the DNA is wrapped in a complex of DNA, histone proteins and RNA called chromatin. This structure presents a basic unit denominated nucleosome, which consists of superhelical DNA (147 base pairs) that rolls up an octamer of four different core histone proteins (two each of H2A, H2B, H3 and H4) in 1.75 turns. It has been shown that modifications of the chromatin structure (boosts or alters) changes gene expression, amongst others of genes involved in the biosynthesis of secondary metabolites. Structurally, chromatin represents an obstacle that complicates access of DNA-binding factors to their corresponding binding regions. According to the compaction level, chromatin can be in a dense (heterochromatin) or relaxing (euchromatin) state. These compaction levels are regulated by post-translational modification of the histone proteins by acetylation, methylation, ubiquitination, ethylation, propylation, butylation and phosphorylation events. Regions that display low transcriptional activity have been associated with the heterochromatic conformation. In contrast, the euchromatic conformation is present in regions with abundant coding sequences and is usually highly active during transcription. Such regions are also linked with

hyper-acetylated nucleosomal histones. Likewise, it has been reported that methylation of H3K9, H3K27 and H4K20 are typical markers of the heterochromatin, while in euchromatin methylation occurs at H3K4 (Brosch *et al.*, 2008; Strauss and Reyes-Dominguez, 2011; Gacek and Strauss, 2012; Spraker and Keller, 2014; Rutledge and Challis, 2015).

Histone methylation, acetylation and sumoylation. As mentioned above, LaeA influences secondary metabolite production through chromatin modification. The methylation state of H3K9 has been correlated with heterochromatin protein A (HepA), since this protein needs the di- and tri-methylation of H3K9 for binding to chromatin and to form heterochromatin. Deletion of LaeA allows the unobstructed binding of HepA to the *AlfR* promoter, thereby affecting the expression of the sterigmatocystin pathway. The deletion of the methyltransferase encoding *clrD* and *ezhB* genes in *Epichloe festucae*, that act on H3K9 and H3K27, respectively (in axenic culture), results in the activation of the ergot alkaloids and lolitrem BCGs. These compounds are necessary to establish a symbiotic association with the plant *Lolium perenne*. Compass (complex of proteins associated with Set1) which methylates H3K4 in yeast, also impacts secondary metabolism in filamentous fungi. The deletion of one of its components (*cclA*) in *A. nidulans* allowed the activation of a cryptic BCG and the production of emodin (Palmer and Keller, 2010; Gacek and Strauss, 2012; Chujo and Scott, 2014; Netzer *et al.*, 2015; Deepika *et al.*, 2016). Likewise, in *Fusarium fujikuroi* and *Fusarium graminearum*, the deletion of *cclA* caused the overproduction of secondary metabolites derived from BCGs close to the telomeres, but this seems to relate to a H3K4 methylation independent mechanism (Studt *et al.*, 2017). Other types of histone modification may alter the chromatin landscape, such as acetylation which is a reversible process governed by two antagonist enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). Active transcription is usually associated with histone acetylation, although recently the deacetylation of histones has been shown to cause activation of genes (Brosch *et al.*, 2008). Usually, histones are acetylated by several complexes with acyltransferase activity, such as Saga/Ada and NuA4. In *A. nidulans* a chromatin immunoprecipitation (ChIP) analysis revealed that GcnE and AdaB, the catalytic subunits of the complex Saga/Ada, are needed for acetylation of histone H3 (Deepika

et al., 2016). Indeed, the interaction between *A. nidulans* and *Streptomyces rapamycinicus* can be linked to a GcnE dependent increase in the acetylation of H3K14 that shields the promoters of the orsellinic acid BGC. The Saga/Ada complex is a key player in the induction of the penicillin, terrequinone and sterigmatocystin BGCs (Nutzmann *et al.*, 2011; Brakhage, 2012). In contrast, deletion of *hdaA* (encoding a HDAC) in *A. nidulans* resulted in major changes in the metabolite profile (Rutledge and Challis, 2015). HdaA is a class 2 histone deacetylase involved in the regulation of BGCs that are located near the telomeres, such as the penicillin and sterigmatocystin clusters in *A. nidulans*. Indeed, deletion of *hdaA* gene results in the increased and early gene expression of these two BGCs, and the production of the corresponding secondary metabolites. In *A. fumigatus*, the *hdaA* gene is involved in growth and production of secondary metabolites, and the deletion of this gene increases the production of many secondary metabolites while it causes a reduction of gliotoxin production. In contrast, HdaA overexpression shows the opposite effect.

HDACs are ubiquitously distributed in filamentous fungi, and therefore HDAC inhibitors can be used to improve the synthesis of natural products by epigenome manipulation (Shwab *et al.*, 2007; Lee *et al.*, 2009). For instance, the metabolite profile of *Cladosporium cladosporioides* and *A. niger* underwent a significant change when these strains were exposed to suberoylanilide hydroxamic acid (SAHA), a HDAC inhibitor, allowing the detection of two new compounds, cladochrome and nygerone A, respectively (Rutledge and Challis, 2015). An exploratory analysis performed in twelve fungi treated with different types of DNA methyltransferase and histone deacetylase inhibitors, revealed the production of new secondary metabolites but also the elevated amounts of known compounds (Williams *et al.*, 2008). In this respect, the chromatin state can directly influence the binding of transcription factors, and thereby modulate expression (Palmer and Keller, 2010; Macheleidt *et al.*, 2016). It has been hypothesized that histone sumoylation may modulate secondary metabolite production. This process is mediated by a small protein termed SUMO (small ubiquitin-like modifier) that shares structural similarity to the ubiquitin protein. In *A. nidulans*, deletion of the *sumO* gene enhanced the production of asperthecin, whereas synthesis of austinol, dehydroaustinal and sterigmatocystin was reduced. Although the molecular mechanism still needs to be elucidated, it is

thought that sumoylation acts at several levels, such as on epigenetic regulators (COMPASS, Ctr4, SAGA/ADA and HDACs) or at the level of transcriptional regulators (Brakhage and Schroeckh, 2011; Spraker and Keller, 2014; Wu and Yu, 2015).

Other targets for regulation. Secondary metabolites produced by fungi can be toxic for themselves. Thus, it is not surprising that fungi have detoxification mechanisms. One of these mechanisms is toxin excretion by transporters, which are membrane proteins whose genes often localize to the BGCs. Transporters may belong to different protein families but the major facilitator superfamily (MFS) and ABC superfamily are most commonly encoded by BGCs (Keller, 2015). Since biosynthesis of secondary metabolites may take place in different cell compartments, also intracellular transport may be evident (Kistler and Broz, 2015). Despite their assumed biological importance, the deletion of transporter genes from the BGCs often does not impact secondary metabolite production. For instance, deletion of the *A. paraisticus aflT* gene, that encodes a MFS transporter, does not result in reduced aflatoxin excretion, despite the fact that *aflT* belongs to the aflatoxin BCG and its expression is regulated by a specific transcription factor, AflR, of the pathway. Probably, this protein is redundant, and other transporters may participate in excretion, detoxification of self-defense. In *A. fumigatus*, GliA facilitates the excretion of gliotoxin. Similarly, the *tri12* gene contained in the trichothecene BGC encodes for a membrane protein required for the biosynthesis of trichothecene and virulence of *Fusarium graminearum* on wheat crops (Chang *et al.*, 2004; Menke *et al.*, 2012; Wang *et al.*, 2014; Keller, 2015). Often, however, the deletion of the transporter gene in BGCs has no effect on production. Possibly, these metabolites are also recognized by other promiscuous transporters, or transporters that are not part of the BGC (Keller, 2015). For example, ZRA1 of *Gibberella zae*, whose gene is not localized to the zearalenone BGC, impacts zearalenone production. However, the expression of the *zra1* gene is regulated by the transcriptional factor ZEB2, whose gene localizes to the corresponding BGC (Lee *et al.*, 2011). Also, the penicillin BGC of *P. chrysogenum* lacks a transporter gene whereas export of penicillin occurs against the concentration gradient, probably through the activity of multiple transporter proteins (van den Berg *et al.*, 2008; Kistler and Broz, 2015).

IV.3 GENETIC ACTIVATION OF BGCS

Several approaches have been used to activate the expression of cryptic BGCS in a targeted manner. Usually, this is achieved by manipulation of pathway-specific regulatory genes, or by replacing endogenous promoters for inducible systems (Rutledge and Challis, 2015). The various approaches are described following and summarized in Figure 3.

Gene deletion. It is a classical strategy that consists in the abolishment of the expression of a certain gene by its elimination whereupon the impact on the metabolite profile is examined by HPLC or LC-MS. A major limitation of this approach is that it can only be used in BGCS that are not totally silenced under laboratory condition. Using this strategy, it was possible to elucidate the highly branched biosynthetic pathway for the synthesis of roquefortine in *P. chrysogenum* (Ali *et al.*, 2013; Ries *et al.*, 2013; Deepika *et al.*, 2016). Likewise, this approach can be used to remove transcriptional repressor genes, as in the case of TetR-like pathway-specific repressor proteins, whose deletion induced the production of gaburedins in *Streptomyces venezualae* (Rutledge and Challis, 2015). Global regulators, such as LaeA have also been targeted using this strategy (Chiang *et al.*, 2009).

Promoter replacement. Another method concerns the replacement of the endogenous promoter of the gene(s) in a BGC by a strong constitutive or inducible promoter. For instance in *A. nidulans* replacement of the native promoter of the *scpR* gene (secondary metabolism cross-pathway regulator) for the inducible promoter of alcohol dehydrogenase *AlcA* induced the expression of a silent cluster that contained two NRPS genes (*inpA* and *inpB*) and *scpR* itself. Additionally, it also led to the expression of the asperfuranone BGC, which is normally silent (Bergmann *et al.*, 2010; Yin and Keller, 2011; Lim *et al.*, 2012).

Overexpression of a specific or global regulator. This approach is one of the most used strategies to turn on cryptic BGCS, since a change in expression level of a regulator may boost the expression of a whole cluster. Usually, this strategy is applied in combination with the *promoter replacement* approach. Using this strategy, i.e., overexpression

of the transcription activator ApdR under control of the alcohol dehydrogenase promoter *alcAp*, it has been possible to induce the expression of a hybrid PKS-NRPS BGC in *A. nidulans*. This resulted in the production of aspyridones A and B (Bergmann *et al.*, 2007). Similarly when the global regulator FfSge, which is associated with vegetative growth of *Fusarium fujikuroi*, is overexpressed, some BGCs are forced to express under these unfavorable conditions (low nitrogen concentrations) leading to the identification of the corresponding products (Michielse *et al.*, 2015).

Modification of the chromatin landscape. Many fungal BGCs are located in distal regions of the chromosomes. In these rich heterochromatin regions, transcription of the BGCs can be activated by epigenetic regulation. Therefore, the encoding genes of proteins that influence histone modification are prime targets, although these modifications can also be achieved by chemical treatment (Williams *et al.*, 2008; Brakhage, 2012).

Heterologous expression and Refactoring. Due to the broad range of molecular tools available to express heterologous pathways in yeast, several attempts have been undertaken to express NRPS and PKS gene with the remainder of the pathway in yeast. However, the main obstacle is the large size (>40kb) of the DNA fragment that needs to be cloned, the effective activation/maturation of the expressed enzymes, and the toxicity of the produced compounds. Alternatively, fungi may be used as platform organism, as for instance it was demonstrated with the reconstruction of the citrinin gene cluster of *Monascus purpurea* in *A. oryzae*. However, the revolutionary introduction of new genetic tools, such as CRISPR/Cas9 offers more effective solutions to express specific BGCs. Such methods can contribute to product identification but also to the production of unique compounds by introduction of specific tailoring enzymes. These are the main strategies that are used for the activation of silent BGCs or for the modification/redirection of known biosynthetic pathways in order to increase product diversification (Spraker and Keller, 2014; Rutledge and Challis, 2015; Smanski *et al.*, 2016; Weissman, 2016)

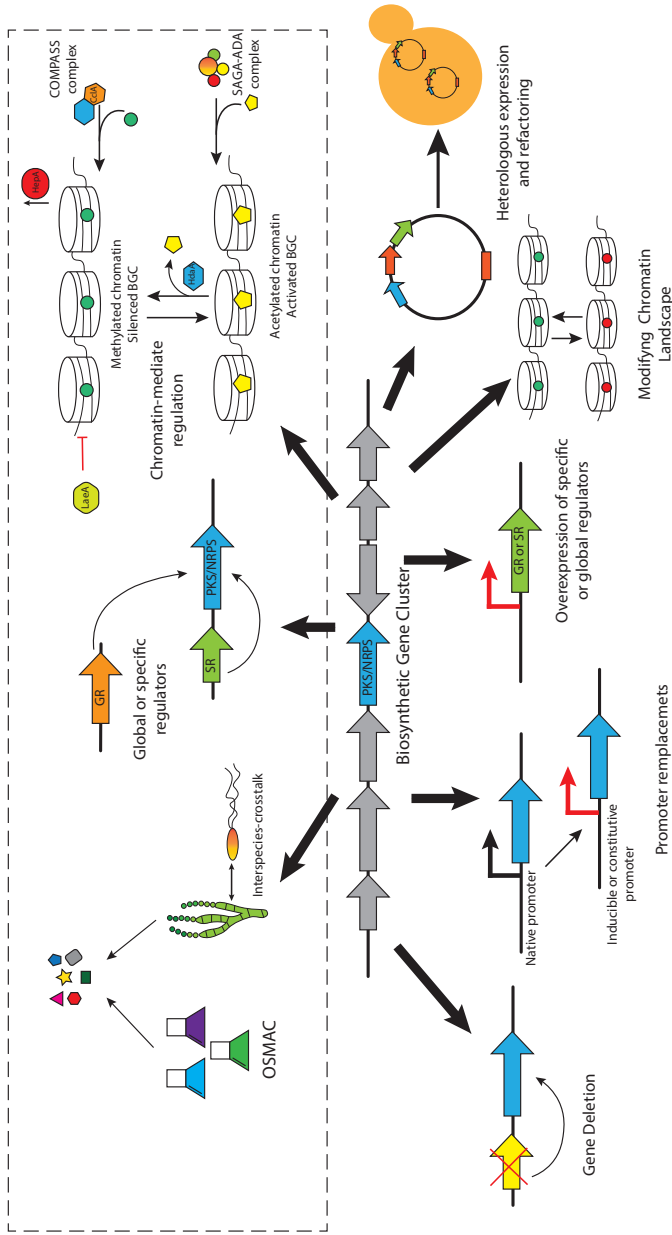


Figure 3. Regulation and strategies to activate silent clusters. For details see the text. Dotted square delimited the secondary metabolite regulation in fungi, that is normally used to induce the expression of secondary metabolites, and employed in genetic engineering. Adapted from (Bode *et al.*, 2002; Brakhage and Schroeckh, 2011; Brakhage, 2012; Spraker and Keller, 2014; Reen *et al.*, 2015; Rutledge and Challis, 2015; Deepika *et al.*, 2016; Smanski *et al.*, 2016).

V. SCOPE OF THE THESIS

This thesis examines the activation of the cryptic sorbicillin gene cluster of an industrial strain of *Penicillium chrysogenum* to elucidate the sorbicillin biosynthesis pathway and regulation mechanism.

Chapter 1 presents an overview of secondary metabolism in filamentous fungi, describes the domain organization of core secondary metabolite enzymes in particular polyketide synthases, and lists main strategies to activate the expression of so-called silent BGCs. In addition, it discusses regulation mechanisms in biosynthetic pathways.

Chapter 2 describes the identification of a polyketide synthase (SorA) involved in sorbicillin biosynthesis in *P. chrysogenum*. Through the repair of a critical point mutation in the *sorA* gene, acquired during the CSI program, sorbicillin production was restored.

Chapter 3 describes the elucidation of the sorbicillin biosynthetic pathway through individual deletion of each gene of the BGC involved in this process. Through metabolic profiling of individual mutants the pathway was deduced, and a novel auto-induction mechanism for regulation of the sorbicillin pathway was resolved.

Chapter 4 shows the effect of the HdaA orthologue in the expression of secondary metabolite associated genes. Using a transcriptional and metabolic profiling, it was demonstrated that HdaA has a broad impact in the regulation of the secondary metabolism, a direct influence in the spore pigmentation and gives evidence of a cross-talk event between gene clusters.

Chapter 5 presents an exploratory analysis of seven transporter protein candidates putatively involved in the export of penicillin.

VI. REFERENCES

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